

Experiences with Some Toxic and Relatively Accessible Heavy Metals on the Survival and Biomass Production of *Amphora costata* W. Smith

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Amphora costata W. Smith 1853 is a down thrown diatom species and also known as metal corrosive ship-fouling organism. *A. costata* was isolated from Alang ship breaking yard, Alang and evaluated the toxicity tolerance and growth responses of the cultures exposed to different doses of toxic and relatively accessible heavy metals, such as Fe, Mn, Cd, Co, Cu, Zn, Ni, and Pb in the constantly monitored laboratory culture conditions. The strongest toxic effect was observed on *A. costata* exposed to Cd even at relatively low concentrations as compared to other metals. The following trend of decreasing order of toxicity i.e. Cd>Zn>Ni>Co>Pb>Cu>Fe was observed, when they were exposed to equal concentration and expose time.

Key Words: *Amphora costata*, benthic diatoms, heavy metal, micro algae, toxicity

INTRODUCTION

A. costata W. Smith, 1853 is a benthic and marine pennate diatom also well known as a fouling diatom species. The characteristics of this organism; strong dorso-ventral frustule has raphe systems which lie close to the ventral margins of the valve. It is also well known as a fouling diatom species. It is observed, exposed to high heavy metal levels in its habitat, polluted sediment or ship hull. To protect ships from this kind of biofouling organisms, bottom of ships are coated with the paints containing different toxic heavy metals such Hg, Cu, Cd, Ni and Pb. *A. costata* is also a notorious producer of bio-film contains extra-cellular polymeric substances (EPS) that protects the cell from the toxic effects of heavy metals.

Responses to different heavy metals treated with numerous representatives of the marine/freshwater phytoplankton includes their growth characteristics, morphological and physiological changes and other phenomenon in *insitu* and *invivo* conditions are published by different workers such as on morphology (Joux-Arab et al 2000), growth rate (Brown et al. 1988), several other physiological processes (Brand et al. 1983, 1986) of benthic

and/or planktonic microalgae in laboratory conditions and as well as lakes (Munawar and Legner 1993), Bays (Ahner et al. 1994; Luoma 1998), coastal waters (Ahner et al. 1997; Bu-Olayan et al. 2001) and open waters (Magi et al. 2005).

Taking into account the toxicity effects on micro algae depends on a number of factors, such as solubility product constant of the respective heavy metal, bio-available free ions, initial concentration of metal as compared to test organism and time of exposure are responsible for the heavy metal interaction with cells in the aquatic environment, we have tried to find out the extreme conditions that can be tolerated by the test organism for long time exposure to individual heavy metal contaminated solutions, via survival and growth responses in relation to chlorophyll *a* production by inhibiting or inducing the culture biomass and re-establishment of its population (succession) in laboratory culture conditions.

MATERIALS AND METHODS

Isolation of Test Organism

The strain of *A. costata* was isolated from the coastal seawater of Alang, Bhavnagar (India). Alang (Lat 21°18' to 21°22' N and Long 70°15' to 72°10' E) ship breaking yard is the largest ship breaking yard in the world.

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Statistical output from its starting of dismantling activities from February 1983 to till January 2005 was 4135 ships with a 30.06 million MT LDT (light dismantling tonnage) were scrapped (http://www.gmbports.org/alang_statsrecyc.htm, visited on 14.03.2006). This area is highly polluted by heavy metals due to ship scrapping activities at Alang ship breaking yard, Alang (Marine Pollution Bulletin News 1998, 2000). The physiochemical parameters and heavy metal contaminations of the coastal area of Alang Ship breaking yard area has been reported in different time as published research paper (Tewari *et al.* 2001; Mandal 2004). The test organism was identified and maintained laboratory culture by consulting different standard methods and literature (Stein 1975; Desikachary 1988,1989; Peragallo 1965).

Media preparation and Culture

To isolate the test organism and to maintain its laboratory culture the established f/2 (Stein 1975) culture media was used with a minor modification. To prepare the f/2 culture media artificially prepared seawater (Berges and Franklin 2001) was used instead of filtered natural seawater to avoid an unknown metal contamination in natural seawater sources.

Experimental conditions

To execute the experiment, 10 round bottom culture tubes (1.8 cm diameter) containing 10 ml of media with different concentrations of specific heavy metal, namely: 0.2, 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 10.0 and 15.0 mg L⁻¹ including control (0 mg L⁻¹) were used. Three different sets of experiments were set-up depending on the duration of harvesting periods, namely: 10 days, 20 days and 30 days for each metal. The culture tubes were well plugged and sterilized at 15psi/121°C for 20 minutes within an automated autoclave (Tomy, Model No. SS 325). Inoculations were done under the sterilized condition using laminar flow (Sanyo-Bioclean Bench Model No. MCV-13BSF). All the culture tubes containing inoculums were kept in slightly tilt (15°) position on specially made white colored wooden platforms. The wooden platforms itself were kept on a shaker for keeping the culture unrest. After 10 days interval, specific cultures were harvested for biomass estimation. All experiments were carried out in double and the average values were used for graphical presentation in Figs 1-8.

Biomass measurement

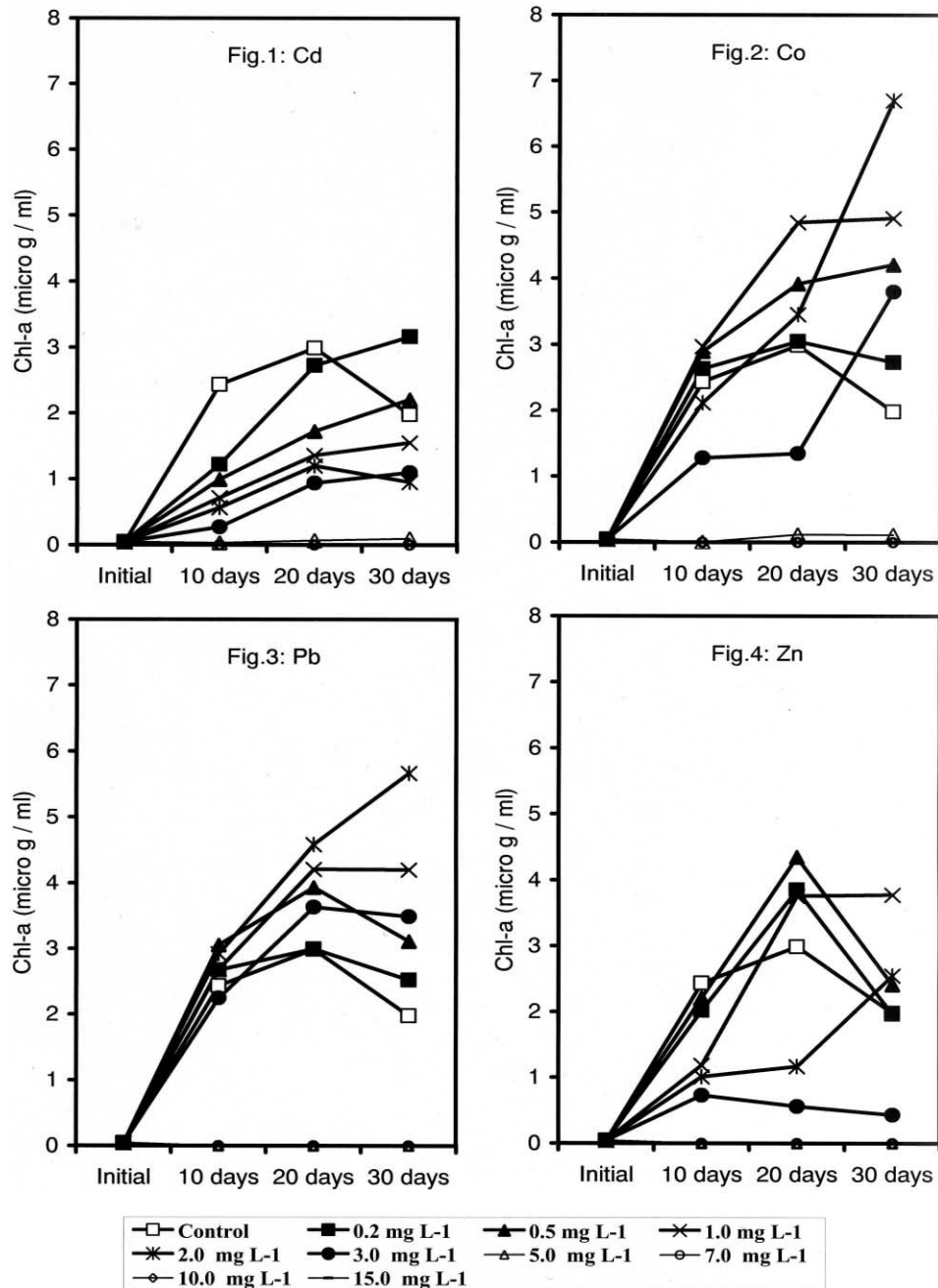
Chlorophyll a is used as an algal biomass indicator

(Clesceri *et al.* 1998). Growth responses were studied by evaluating increase of biomass in terms of µg chlorophyll a/ml. Even though all cultures were maintained on a continuous shaker, in some cases, a very few number of culture cells were observed to stick on the glass wall (ring formation) at the air-water (culture media) interface area. That's why biomass evaluation through chlorophyll a estimation gave more accuracy (> 99.9%) than enumeration. To avoid loss of cells due to attachment, total culture was filtered through nitro-cellulose filter paper (porosity 0.45 µ) and the filter paper was then put into the same culture tube and 10 ml of 90% acetone was added to extract chlorophyll a pigment from the filtered culture as well as attached cell with the culture tube if any. The absorbance of chlorophyll a pigment were measured in a Shimadzu spectrophotometer (1201 UV-VIS) using the specified wave length and quantified by the given international standard equations for pigment analysis (Strickland and Parsons 1972).

RESULTS

The effects of different heavy metals on *A. costata* as regards the biomass increase over time, also indicating the upper limits of tolerance are depicted in Figs 1-8. The comparative effects of the heavy metal treatments of the following doses i.e. 0.2, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg L⁻¹ are depicted in Figs 9-11 as all culture containing more than 5.0 mg L⁻¹ of heavy metals showed total inhibition of biomass production.

Cadmium was the most toxic heavy metal as compared to other seven metals and showed the strongest effect on the growth response of *A. costata*. The toxic effects of Cd on the survival and biomass production were observed at relatively very low concentration even at 0.2 mg L⁻¹ and total inhibition of biomass production of *A. costata* was observed at 5.0 mg L⁻¹ Cd and above. Zn showed the second highest toxicity on the survival and biomass production of *A. costata* following Cd. Zn brought stress on biomass production in first 10 days in all culture and there was total inhibition of growth more than 3.0 mg L⁻¹. The optimum concentrations of different heavy metals, which were tolerated by *A. costata* as well as showed maximum growth of *A. costata* in long term (30 days) exposure were 0.2 mg L⁻¹ Cd (Fig. 1), 2.0 mg L⁻¹ Co (Fig. 2), 2.0 mg L⁻¹ Pb (Fig. 3), 1.0 mg L⁻¹ Zn (Fig. 4), 2.0 mg L⁻¹ Cu (Fig. 5), 2.0 mg L⁻¹ Ni (Fig. 6), 2.0 mg L⁻¹ Mn (Fig. 7) and 1.0 mg L⁻¹ Fe (Fig. 8). The maximum tolerance limits for the respective heavy metals were 5.0

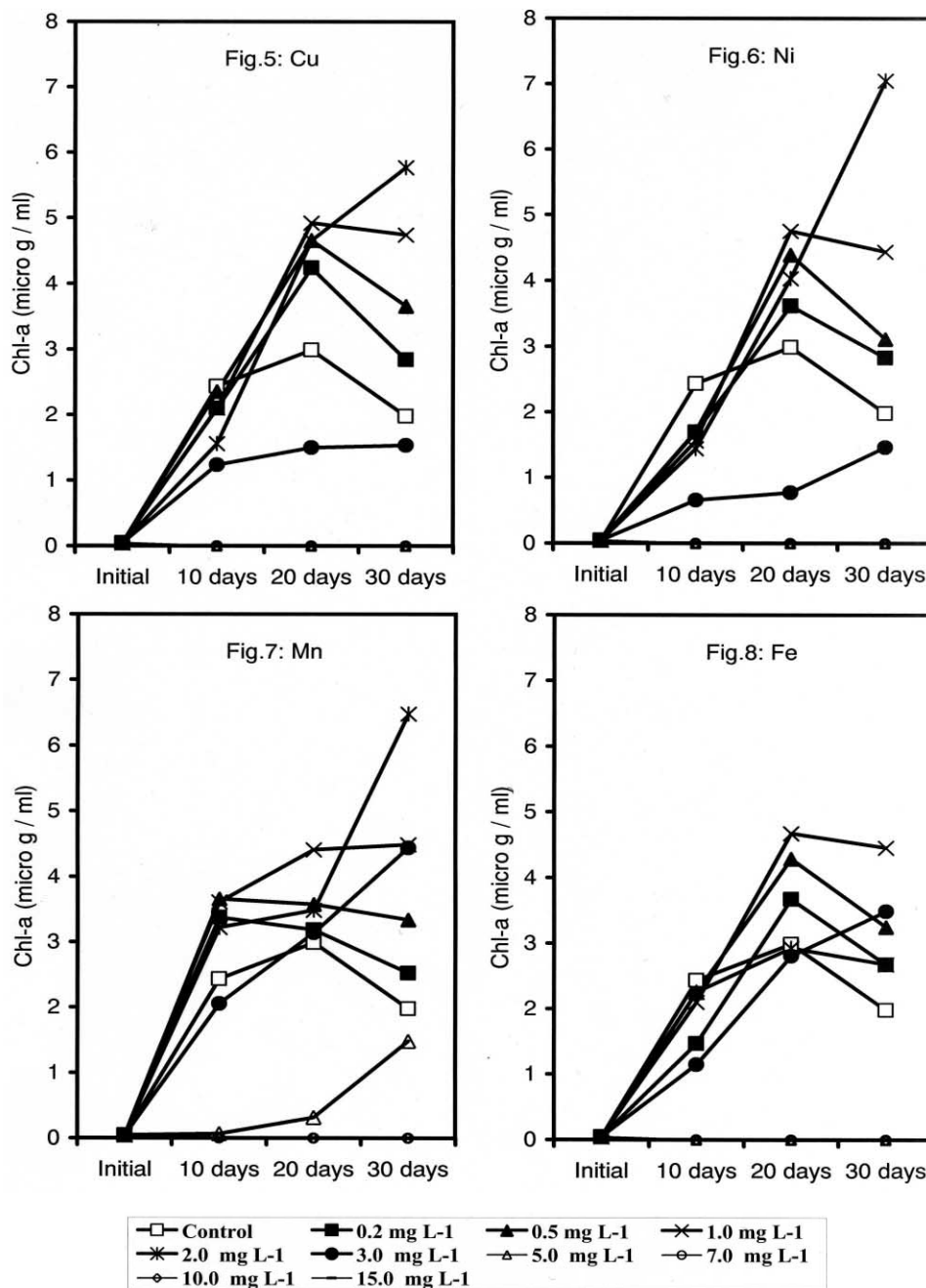


Figs 1-4. Increase or decrease of Biomass of *Amphora costata* in the f/2 growth media treated with different doses of Cd, Co, Pb and Zn.

mg L⁻¹ Cd, 5.0 mg L⁻¹ Co, 3.0 mg L⁻¹ Pb, 3.0 mg L⁻¹ Zn, 3.0 mg L⁻¹ Cu, 3.0 mg L⁻¹ Ni, 5.0 mg L⁻¹ Mn and 3.0 mg L⁻¹ Fe. The essential heavy metals such as Co, Zn, Cu, Mn and Fe also showed inhibitory growth of *A. costata*, as compared to control or total mortality when they were present at higher concentration, i.e. 5.0 mg L⁻¹ and/or above (Figs 1-8).

In general, during the first 10 days of culture, the inhibited growth in relation to biomass production as compared to control were observed in all cultures con-

taining 2.0 mg L⁻¹ and higher concentration of Cd, Zn, Cu, Ni and Fe and containing 3.0 mg L⁻¹ and higher concentration of Co, Pb and Mn (Fig. 9). Whereas after 20 days of cultivation *A. costata* showed less biomass production as compared to control in all cultures containing 0.2 mg L⁻¹ and higher concentration of Cd, 2.0 mg L⁻¹ and higher concentration of Zn and containing 3.0 mg L⁻¹ and higher concentration of Co, Cu, Ni and Fe (Fig. 10). In 30 days of culture, most of the cultures overcame the stress condition below 5.0 mg L⁻¹. Even though, after



Figs 5-8. Increase or decrease of Biomass of *Amphora costata* in the f/2 growth media treated with different doses of Cu, Ni, Mn and Fe.

30 days of culture, less biomass production as compared to control were observed in 1.0 mg L⁻¹ and higher concentration of Cd and containing 3.0 mg L⁻¹ and higher concentration of Zn, Cu, and Ni (Fig. 11).

DISCUSSION

Our observations on the effects of toxicity of the toxic and relatively accessible heavy metals in relation to the survival and biomass production of *A. costata* were var-

ied depending upon their initial concentrations, which were added with the medium and the time of exposure.

Cadmium is a non-essential and toxic trace element and also known to be a potential toxicant at extremely low concentrations. It ranks close to lead as bio-toxic agents and can induce a wide range of toxicity (Mukherji and Sharma 1987). Growth inhibition in response to increasing cadmium in the medium has also been reported earlier by Visviki and Rachlin (1991) and Thompson and Couture (1991). For example, *Chlamydomonas rein-*

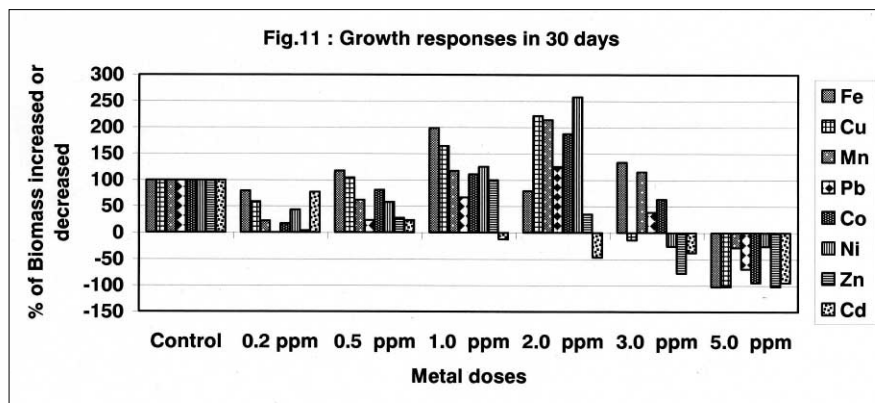
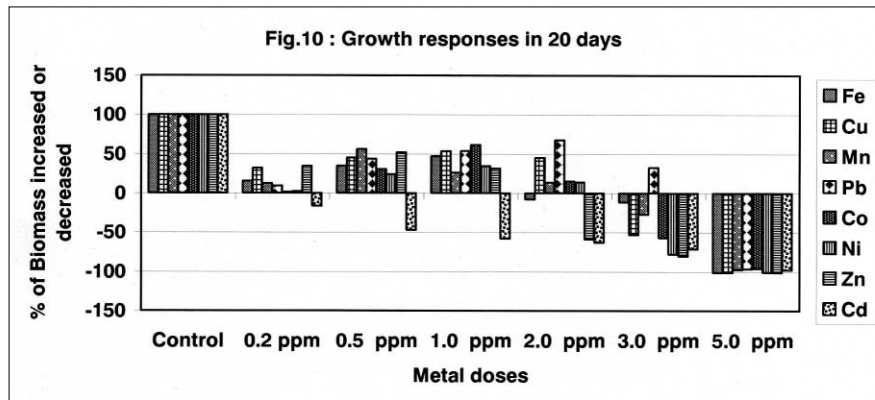
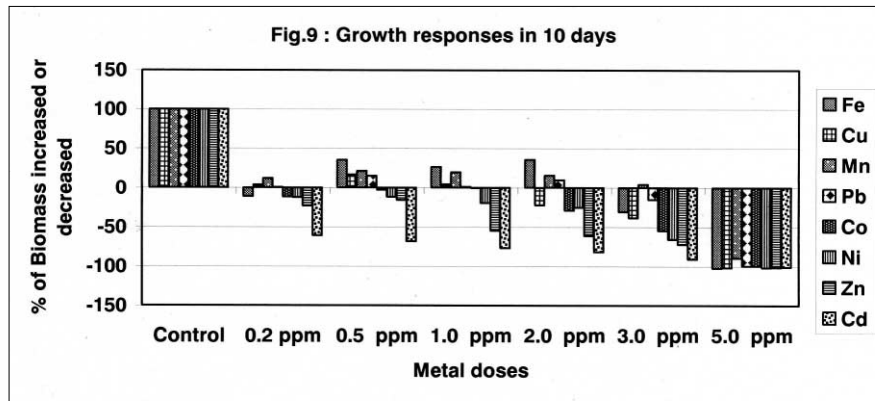


Fig 9-11. *Amphora costata* overcomes the environmental stresses due to long time exposure in the f/2 growth media treated with different heavy metal doses for 10 days, 20 days and 30 days old culture. As in higher concentration like 7, 10 and 15 ppm the inoculum of *Amphora costata* were completely dead (100%) and that's why those concentrations are not considered in these comparative charts.

hardtii and *Pleurochrysis cartae* are reported as gradually reduced growth rate with increase of Cd concentration above 3×10^{-8} M (Chris and Neil 1999). Cd is found the most toxic heavy metal, because it might have brought devastating effects on the growth responses of *A. costata* in different way, such as morphological and physiological changes. Torris *et al.* (1998) reported decrease of ATP content and ultra structural changes were also observed in *P. triconutum* in 5.0 mg L^{-1} or higher Cd contained

media. Nickel is a non-essential trace metal and found as toxic heavy metal in relation to growth response of *A. costata*. The suppressive impact on growth by Ni on *A. costata*, might be due to the ion-ion competition of essential and non-essential ions. Zinc is an essential trace element. It is required in forming complexes, such as zinc fingers in DNA and as a component in cellular enzymes (Nies 1999). The strong toxic effect of Zn metal also reported by Stauber and Florence (1990) that $65 \mu\text{g Zn}$

L⁻¹ halved the cell division rate to the marine diatom *Nitzschia closterium*. Copper is an essential trace element and is used by cells in very small quantities in cellular enzymes. Copper toxicity was reported as most toxic within Copper, Nickel, Zinc and Lead by Danilov and Ekelund (2001) on photosynthetic efficiencies of *Chlamydomonas reinhardtii* on short term exposure to 0.5 mg L⁻¹ and higher. Cobalt is an essential element for growth and biomass production for marine micro algae. Co is a co-factor in biomolecules, vitamin B₁₂ (cyanocobalamin) which is required for N₂ fixation and the growth of many algal (diatom) species (Martin and Gordon 1988). Even though, the authors are not much aware about any previous report on cobalt and Mn toxicity to Marine phytoplankton, but they have experienced an inhibitory effect on biomass production as compared to control at 2.0 mg L⁻¹ of Co, 3.0 mg L⁻¹ of Mn and higher concentration. Lead showed less toxic effect as compared to Cd, Ni, Zn and Co in lower concentration than 2.0 mg L⁻¹ for the first 10 days. Danilov and Ekelund (2001) reported lead has a stimulatory effects on photosynthetic efficiency of *Chlamydomonas reinhardtii* in gradually increased lead concentration from 0.1 to 2.0 mg L⁻¹, which might be responsible for higher biomass production in relatively lower concentration from 0.2 to 2.0 mg L⁻¹ as compared to control.

According to the findings of the present study, it leads to the assumption that the interaction between extra cellular polymeric substances (EPS) production by *A. costata* in the environmental stress condition generated by heavy metal doses and bioavailability of heavy metals is principal cause to tolerate/overcome the toxicity effects of heavy metals to some extent and survival of the test organism. EPS are known for their ability to bind a wide range of heavy metals, including Pb, Co, Cu, Fe and Cd etc (Bhaskar and Bhosle 2005; Rijstenbil and Gerringa 2002; Florence *et al.* 1992). EPS can bind with free metal ions and reduced the available free ions in the aquatic environment due to long exposure in metal contaminated solution, which might have lead to reestablish the population after struggling into a adverse condition. Binding of dissolved metal ions from the contaminated solutions by EPS and moved towards depth has been reported by Decho (1990). The dominating role of EPS controlling the accumulation of heavy metals like Cu, Zn, on *Nostoc linckia* (Roth) were also observed by Shnyukova (2005). EPS might have played an important role to reduce lead toxicity as compared to other toxic metal by forming EPS-Lead complex as EPS production

becomes more at the exponential stage (Bhaskar and Bhosle 2005). Even though the test organism was the same, but the toxic effects of heavy metals varied in case of different heavy metals depending upon their concentrations and the age of test culture organism during the growing stage might be due to electronegativity and bioavailability of metal ions.

The above studies envisaged that the trend of the decreasing order of toxicity on survival and biomass production of *A. costata* was following i.e. Cd > Zn > Ni > Co > Pb > Mn > Cu > Fe. We can get a clear idea about the effective metal concentration, which can be used to protect the growth of the test organism on the ship-halls or boat etc. However, the results obtained in laboratory conditions are not directly applicable in the open environments as most of the parameters are uncontrollable in open environment due to metal-metal competition, and metal-organic matters interactions in the open environment, more over marine pollution is also highly concerned in this regard.

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REFERENCES

- Ahner B.A., Morel F.M.M. and Moffett J.W. 1997. Trace metal control of phytochelatin production in coastal waters. *Limnol. Oceanogr.* **42**: 601-608.
- Ahner B.A., Price N.M. and Morel F.M.M., 1994. Phytochelatin production by marine phytoplankton at low free metal ion concentrations: Laboratory studies and field data from Massachusetts Bay. *Proc. Natl. Acad. Sci. USA.* **91**: 8433-8436.
- Berges J.A. and Franklin D.J. 2001. Evolution of Artificial seawater medium: Improvements in Enriched seawater, Artificial water over the last two decades. *J. Phycol.* **37**: 1138-1145.
- Bhaskar P.V. and Bhosle N.B. 2005. Microbial extracellular polymeric substances in marine biogeochemical processes. *Curr. Sci.* **88**: 45-53.
- Brand L.E., Sunda W.G. and Guillard R.R.L. 1983. Limitation of marine phytoplankton reproductive rates by zinc, manganese and iron. *Limnol. Oceanogr.* **28**: 1182-1198.
- Brand L.E., Sunda W.G. and Guillard R.R.L. 1986. Reduction of Marine phytoplankton reproduction rates by copper and cadmium. *J. Exp. Mar. Biol. Ecol.* **96**: 225-250.

- Brown L.N., Robinson M.G. and Hall B.D. 1988. Mechanisms for tolerance in *Amphora coffeaeformis*-internal and external binding. *Mar. Biol.* **97**: 581-586.
- Bu-Olayan A.H., Al-Hassen R., Thomas B.V. and Subrahmanyam M.N.V. 2001. Impact of trace metals and nutrients levels on phytoplankton from the Kuwait Coast. *Environment International*. **26**: 199-203.
- Chris D.P. and Neil M.P. 1999. Effects of Cadmium toxicity on growth and elemental composition of marine phytoplankton. *J. Phycol.* **35**: 293-302.
- Clesceri L.S., Greenberg A.E. and Eaton, A.D. 1998. *Standard Methods for the Examination of Water and Wastewater* (20th Edition). American Public Health Association, Washington.
- Danilov R.A. and Ekelund NGA. 2001. Effects of Cu²⁺, Ni²⁺, Pb²⁺ and Zn²⁺ and pentachlorophenol on photosynthesis and motility in *Chlamydomonas reinhardtii* in short-term exposure experiments. *BMC Ecology* (<http://www.biomed-central.com/1472-6785/1/1>) 1.1
- Decho A.W. 1990. Microbial biofilms in intertidal system: an overview. *Cont. Self. Res.* **20**: 73-153
- Desikachary T.V. 1988 *Atlas of Diatoms- Marine diatoms of the Indian Ocean region Vol. 5*. Madras Science Foundation, Madras.
- Desikachary T.V. 1989. *Atlas of Diatoms- Marine diatoms of the Indian Ocean region Vol. VI*. Madras Science Foundation, Madras.
- Florence T.M., Powell H.K.J., Stauber J.L. and Town R.M. 1992. Toxicity of lipid-soluble copper(II) complexes to the marine diatom *Nitzschia closterium*: Amelioration by humic substances. *Water Research* **26**: 1187-1193.
- Joux-Arab L., Berthet B., and Robert J-M. 2000. Do toxicity and accumulation of copper change during size reduction in the marine pinnate diatom *Haslea ostrearia*? *Mar. Biol.* **136**: 323-330.
- Luoma S.N., Geen A.V., Lee B-G., and Cloern J.E., 1998. Metal uptake by phytoplankton during a bloom in South San Francisco bay: Implications for metal cycling in estuaries. *Limnol. Oceanogr.* **43**: 1007-1016.
- Magi E., Ianni C., Soggia F., Grotti M. and Frache R. 2005. Trace metals speciation in coastal particulate matter for marine environmental studies in Antarctica. *J. Environ. Monit.* **7**: 1287-1294.
- Mandal S.K. 2004. *Studies on the effects of ship scrapping industry wastes on marine phytoplankton at Alang, Gujarat*. A Ph.D. thesis. Bhavnagar University, Bhavnagar, Gujarat, India.
- Marine Pollution Bulletin News. 1998 Toxic waste ships exported for scrap. *Mar. Pollut. Bull.* **36**: 322.
- Marine Pollution Bulletin News. 2000: Protest at scrapping of ship in china. *Mar. Pollut. Bull.* **40**: 1.
- Martin J.H. and Gordon R.M. 1988. Northeast Pacific iron distributions in relation to phytoplankton productivity. *Deep-Sea Research* **35**: 177-196.
- Mukherji A. and Sharma A. 1987. Effects of Cadmium and zinc on cell division and chromosomal aberration in *Allium sativum*. *Curr. Sci.* **56**: 1097-1100.
- Munawar M., and Legner M. 1993. Detection of Toxicity Using Natural Phytoplankton as Test Organisms in the Great Lakes. *Water Pollut. Res. J. of Canada* **28**: 155-176.
- Nies D.H. 1999. Microbial heavy metal resistance. *Appl Microbiol Biotechnol* **51**: 730-750.
- Parsons T.R., Maita Y. and Lalli C.M. 1985. *A Manual of Chemical and Biological Methods for Seawater Analysis*. Pergamon Press, Oxford.
- Peragallo M. 1965. *Diatomees Marines De France - Texte*. A. Asher & Co., Amsterdam.
- Rijstenbil J.W. and Gerringa L.J.A. 2002. Interactions of ligands, metal complexation and availability, and cell responses of the diatom *Ditylum brightwellii* with a gradual increase in copper. *Aquat. Toxicol.* **56**: 115-131.
- Shnyukova E.I. 2005. Accumulation of metal ions by exopolysaccharides of *Nostoc linckia* (Roth) Born. *Et Flach. Int. J. on Algae* **7**: 101-107.
- Stauber J.L. and Florence T.M. 1990. Mechanism of toxicity of zinc to the marine diatom *Nitzschia closterium*. *Mar. Biol.* **105**: 519-524.
- Stein J. R. 1975. *Handbook of Phycological Methods; culture methods and growth measurements*, Cambridge university Press, Cambridge.
- Strickland J.D.H. and Parsons T.R. 1972. *A Practical Handbook of Seawater Analysis*. Bull, 167, Fisheries Research Board of Canada, Ottawa.
- Tewari A., Joshi H.V., Trivedi R H., Sravankumar V.G., Raghunathan C., Kotiwar O.S., Khambhati Y. and Mandal, S.K. 2001. The effect of the ship scrapping Industry and its associated wastes on the biomass production and biodiversity of biota in *in situ* condition at Alang, *Mar. Poll. Bull.* **42**: 462-469.
- Thompson P.-A. and Couture P. 1991. Short-and long term changes in growth and biochemical composition of *Selenastrum capricornutum* populations exposed to cadmium. *Aquat. Toxicol.* **21**: 135-143.
- Torris E., Cid A., Herrero C. and Abalde J. 1998. Effect of Cadmium on growth, ATP content, Carbon fixation and ultrastructure in the marine diatom *P. tricornutum* Bolin. *Water Air Soil Poll.* **117**: 1-14.
- Visviki I. and Rachlin J W. 1991 The toxic action and interactions of copper and cadmium to the marine Alga *Dunaliella minuta*, in both acute and chronic exposure. *Arch. Environ. Con. Tox.* **20**: 271-275.

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